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## **Introduction**

This report covers supplemental research conducted from July 1, 2003 through September 30, 2004 as a no-costs extension of our original award. The major goal of research conducted under this contract was to identify early changes in mitochondrial and neuronal function following exposure to neurotoxins that may contribute to neuronal damage through apoptotic mechanisms. The specific goals of this contract were: 1) to first characterize toxin-induced alterations of respiratory chain redox status and mitochondrial membrane potential in intact functioning neuronal populations; 2) to examine the role of specific intracellular mediators of mitochondrial and neuronal dysfunction including release of mitochondrial cytochrome c, elevation of intracellular calcium, and production of reactive oxygen species (ROS). A final goal was to determine whether changes in mitochondrial function after toxin exposure was consistent with the hypothesis that mitochondria undergo permeability transition of the inner mitochondrial membrane, as a mechanism for initiation of pro-apoptotic death cascades. In this report we provide additional data from research directed toward these goals. These data include: 1) experiments probing mitochondrial involvement in the apoptotic pathway mediated by Poly (ADP-ribose) polymerase (PARP); 2) additional experiments using Enzyme-Dependent Fluorescence Recovery After Photobleaching (ED-FRAP) to monitor citric acid cycle dehydrogenase activity for production of mitochondrial NADH; 3) a method for quantitative assessment of nicotinamide adenine dinucleotide (NADH) concentration in tissue; and 4) additional experiments attempting to indicate toxin-induced mitochondrial permeability transition in organotypic slice cultures using immunohistochemistry and the permeability transition inhibitor Cyclosporin A.

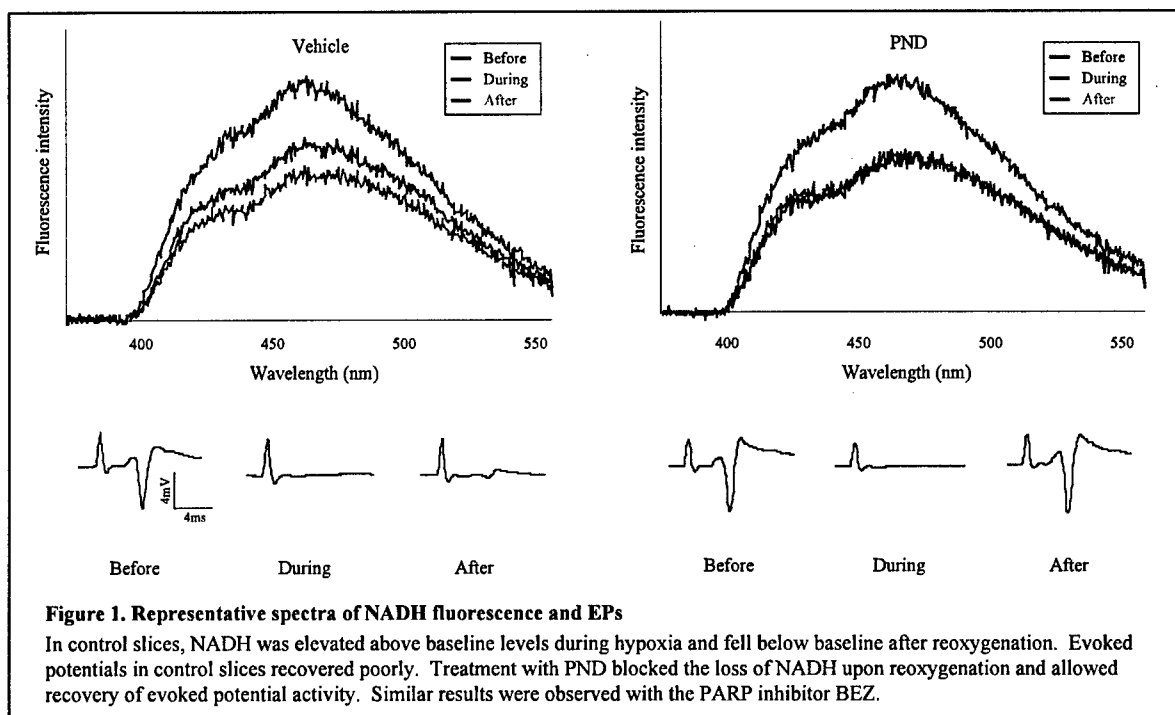
## **Body**

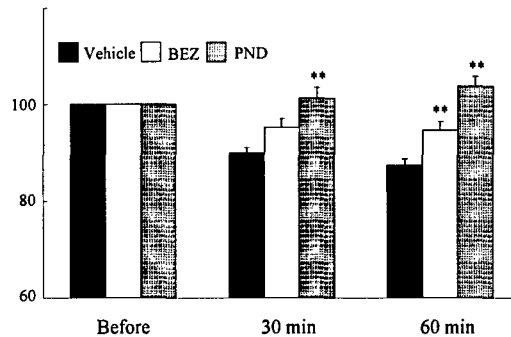
Our previous research clearly demonstrated that toxins inhibiting mitochondrial respiratory chain Complex I caused neuronal death in hippocampal slice cultures and that this death resembled the selective neuronal damage observed in hippocampus following severe hypoxia (Complex IV inhibition, Xu et al., 2003). However, to date we have not been able to demonstrate that this toxin-induced neuronal death resulted from apoptosis, at least through the pathway mediated by mitochondrial permeability transition, release of cytochrome c, and activation of caspase-3. We have thus far been unable to demonstrate mitochondrial depolarization, immunohistochemical evidence of cytochrome c release from mitochondria, or immunohistochemical evidence of caspase-3 activation in acute brain slice preparations from adult rats after exposure to mitochondrial toxins. However, these toxins clearly cause neuronal cell death

### **Poly (ADP-ribose) Polymerase (PARP)**

PARP is predominantly a nuclear enzyme that is selectively activated by nuclear DNA strand breaks. This enzyme transfers ADP-ribose subunits acquired from NAD<sup>+</sup> to a variety of proteins that function to repair DNA damage by a mechanisms that are currently poorly understood. However, activation of PARP leads to rapid depletion of cytosolic NAD<sup>+</sup>, the restoration of which requires ATP and which subsequently leads to

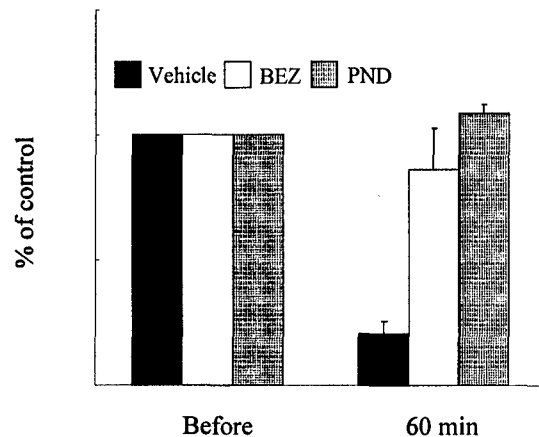
cytosolic ATP depletion that may cause necrotic cell death (see Ha and Snyder, 2000 for review). It has also recently been shown that PARP activation causes the mitochondrial release of apoptosis inducing factor (AIF) that translocates to the nucleus and initiates apoptosis through a mechanism independent of cytochrome c release and activation of caspase pathways (Yu et al., 2002). Moreover, it has also been suggested recently that an intramitochondrial form of PARP may contribute to NAD<sup>+</sup> depletion and cell death following oxidative stress (Du et al., 2003). We have previously shown that mitochondrial Complex IV inhibition (severe hypoxia) resulted in loss of intramitochondrial NADH and that this loss was associated with failure of functional recovery in brain slices (Rosenthal, 1995; Perez-Pinzon, 1998). The loss of intramitochondrial was calcium-dependent (Perez-Pinzon, 1998) and was inhibited with antioxidants (Perez-Pinzon, 1997). In the following experiments, we monitored intramitochondrial NADH by microspectrofluorometry during severe hypoxia in the presence and absence of the PARP inhibitors benzamide (BEZ) or 6(5H)-phenanthridinone (PND) in hippocampal slices (Hagioka et al., 2004). In addition, we monitored electrical activity in subfield CA1 following stimulation of the Schaffer collaterals. Figure 1 shows the effects of PARP inhibition with PND on intramitochondrial NADH and evoked electrical activity.





**Figure 2. Changes in NADH fluorescence.**

PND and BEZ significantly prevented loss of NADH at 60 min after 3 min of anoxia. Before: Before anoxia, 30 min: 30 min after the onset of reoxygenation, 60 min: 60 min after the onset of reoxygenation,  $n = 5$ , \*\*:  $p < 0.05$  vs vehicle.



**Figure 3. Changes in EPs After PARP Inhibition**

PARP inhibitors significantly improved EPs recovery after anoxia. No recovery of EPs was observed in vehicle-treated slices. PND significantly recovered EPs to  $89.7 \pm 18.3\%$  of control. ( $n = 5$ , \*\*:  $p < 0.05$  vs vehicle).

The data indicate that PARP can affect intramitochondrial NADH levels after Complex IV inhibition in brain slices. It is not known yet, however, whether the effect is mediated by nuclear or mitochondrial PARP. We, and others, have shown an increase in ROS formation during re-oxygenation after severe hypoxia or ischemia. ROS production is also stimulated after Complex I inhibition. If ROS production causes DNA damage (either mitochondrial DNA or nuclear DNA), PARP activation could lead to depletion of mitochondrial NADH. Depletion of mitochondrial NADH would occur directly upon stimulation of mitochondrial PARP. However, depletion of mitochondrial NADH might also occur due to depletion of cytosolic NAD due either to the inability to shuttle NAD into mitochondria, or due to inhibition of glycolysis and the provision of energy substrates to the citric acid cycle. Experiments are planned to determine which of these processes is involved, and whether mitochondrial toxin exposure leads to neuronal apoptosis through a PARP-mediated pathway.

#### Citric Acid Cycle Dehydrogenase Activity

In the previous progress report, we showed preliminary data employing a new method for monitoring citric acid cycle dehydrogenase activity in intact cells in brain slices. We have continued to investigate the use of this method in the past year of funding.

**ED-FRAP** – Enzyme Dependent Fluorescence Recovery After Photobleaching is a new method for monitoring the activity of mitochondrial citric acid cycle dehydrogenase activity recently reported by Combs and Balaban, (2001). FRAP is a commonly used method to monitor diffusion rates of molecules in cell membranes in which a fluorescent molecule is photo-bleached and the recovery of fluorescence is dependent upon diffusion of nearby molecules into the membrane. ED-FRAP takes advantage of the fact that NADH fluorescence in cells occurs predominantly in mitochondria and NADH is not freely diffusible or transported into mitochondria. Levels of mitochondrial NADH are determined primarily by activity of citric acid cycle dehydrogenases. Thus when NADH is photo-bleached, the rate of recovery of NADH fluorescence is dependent upon dehydrogenase activity (Combs and Balaban, 2001). It is important also that photobleaching of NADH results in photo-oxidation of NADH to  $\text{NAD}^+$  and not depletion of the intra-mitochondrial pyridine nucleotide pool [Joubert, 2002 #135] so that replenishment of NADH after photobleaching is possible.

**Organotypic Slice Cultures** – NADH measurements and ED-FRAP analysis in organotypic slice cultures was conducted with confocal fluorescence microscopy. Our laboratory has access to a Zeiss 510 Laser Confocal Microscope equipped with a near-ultraviolet (351 nm) laser and FRAP software. The software allows selection of one or more regions of interest (ROIs) which can be independently exposed to high levels of excitation illumination required for photobleaching. Regions outside of the ROIs remain unbleached. An example of an NADH photobleaching experiment showing the bleached CA3 region of an organotypic slice culture, and the accompanying fluorescence quantification are shown in Figure 4.

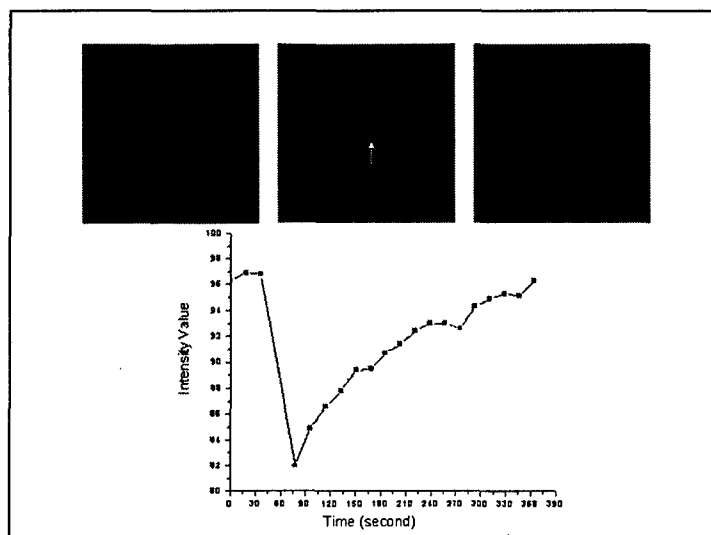


Figure 4. Pseudo-colored confocal NADH fluorescence images acquired from subfield CA3 of a hippocampal slice culture. Images were acquired prior to (left panel), immediately after (center panel), and upon recovery from (right panel) photobleaching. The arrow in the middle image shows the photobleached region of interest immediately after the photobleaching exposure. The graph shows average NADH fluorescence intensity of the region of interest over the time course of the experiment.

We have subsequently conducted experiments to examine ED-FRAP in isolated citric acid cycle reactions, in preparations of isolated mitochondria, and in brain slices.

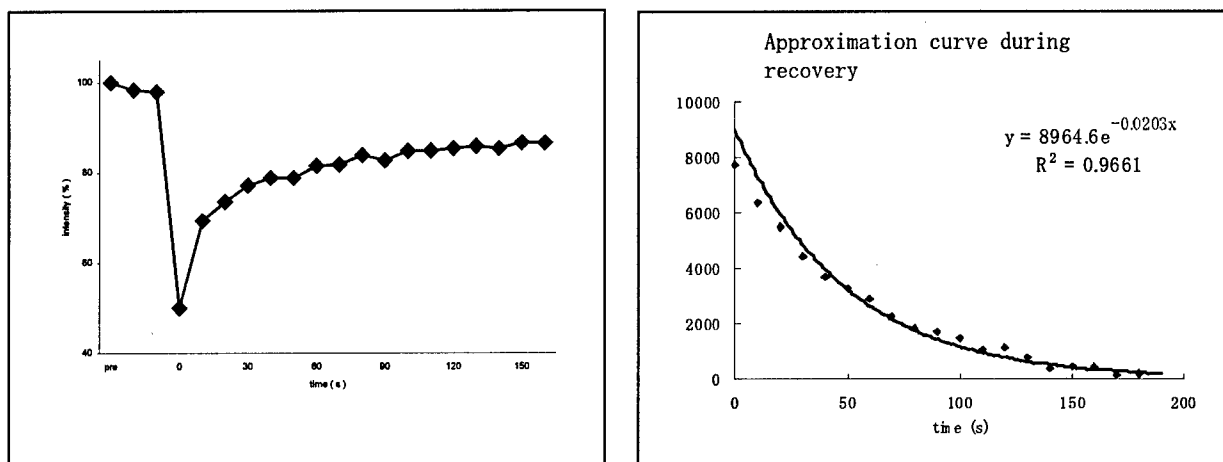
#### 1) Glutamate Dehydrogenase



In the presence of  $\text{NAD}^+$  and glutamate, glutamate dehydrogenase reversibly produces  $\alpha$ -ketoglutarate and NADH according to the following reaction:

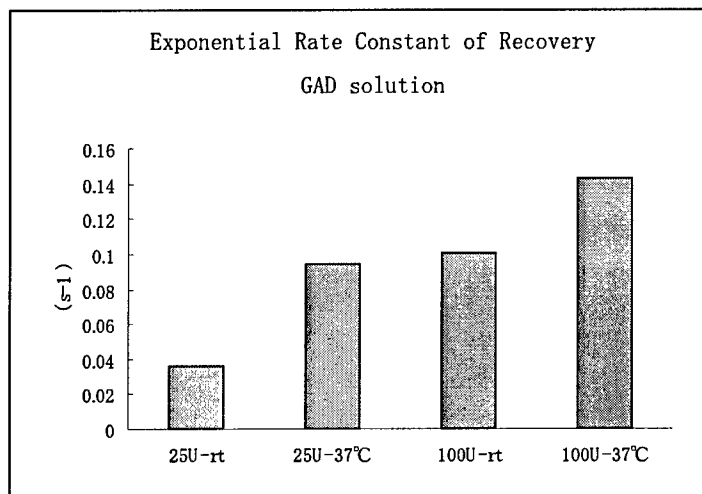


In these experiments, NADH was measured in the reaction medium using microspectrofluorometry. Photobleaching of NADH was accomplished by increasing the excitation exposure from 5 msec (used for recording NADH) to 2 sec (used for bleaching).



**Figure 5.** Left Panel: NADH ED-FRAP from glutamate dehydrogenase reaction in vitro. Right Panel: Calculation of rate constant for recovery of NADH after photobleaching.

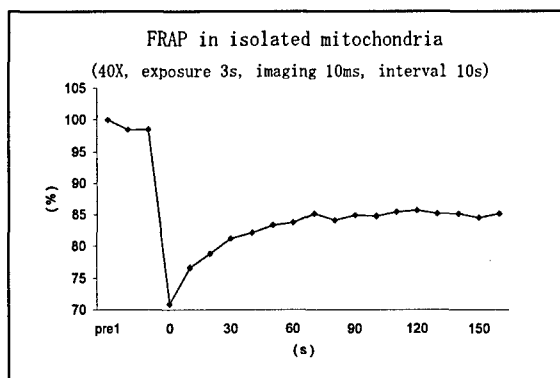
Using this method, we demonstrated that the rate of NADH recovery after photobleaching was dependent upon enzyme activity and temperature as expected of an isolated enzyme reaction. These data are shown in figure 6 below.



**Figure 6.** Effect of glutamate dehydrogenase (GAD) concentration (international units) and temperature (rt = room temperature) on the rate constant of NADH recovery after photobleaching.

## 2) Isolated Mitochondria

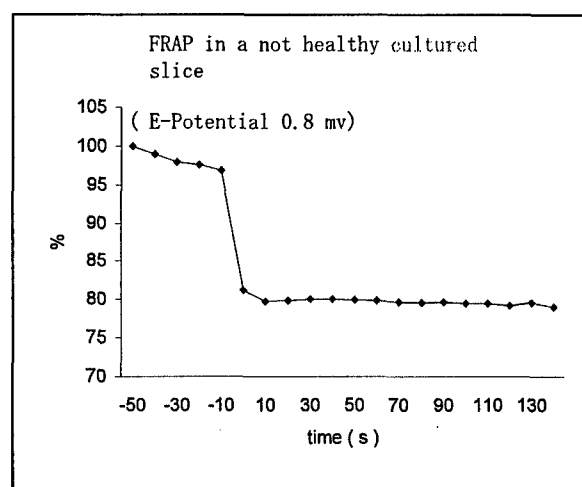
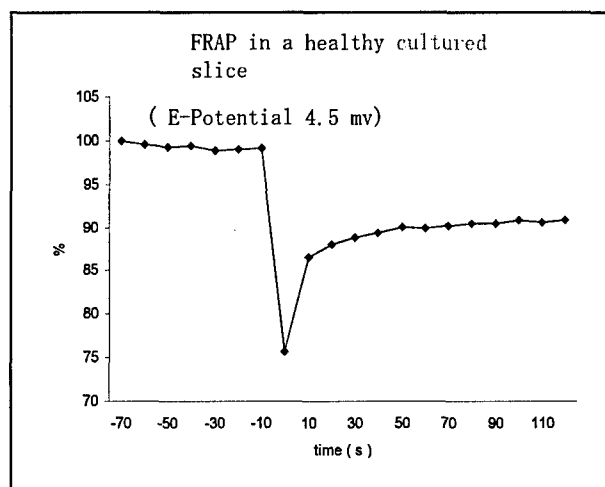
In these experiments, brain mitochondria were isolated by cell fractionation and centrifugation as described in our laboratory by Dave et al., (2001). Mitochondrial NADH was bleached and the recovery of fluorescence was monitored as described above. An example of this experiment is shown below in figure 7.



**Figure 7.** Recovery of NADH after photobleaching in a preparation of isolated mitochondria. Mitochondria were placed in a culture dish on the stage of an upright microscope equipped to provide ultraviolet excitation light of varying intensity and duration. Fluorescence emission was measured by microspectrofluorometry as described previously.

## 3) Brain Slices

The following example shows measurement of NADH ED-FRAP from brain slices. The data also show that NADH ED-FRAP is correlated to the electrophysiological health of the brain slice.



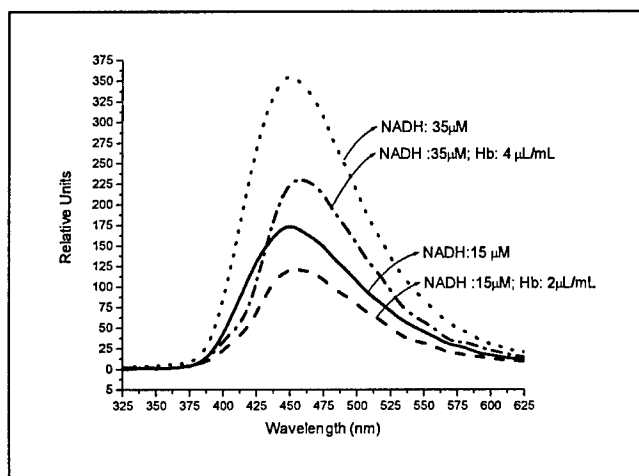
**Figure 8.** Examples of NADH ED-FRAP measurements from two brain slices. The brain slice on the left was considered “healthy” because of a robust evoked potential recorded in hippocampal subfield CA1. The brain slice on the right was considered “unhealthy” because of poor electrical activity. It is possible that the failure of electrical activity in the “unhealthy” brain slice resulted from mitochondrial dysfunction.

### **Quantitative Analysis of Brain NADH in the Presence of Hemoglobin Using Microfiber Spectrophotometry: A Pre-calibration Approach**

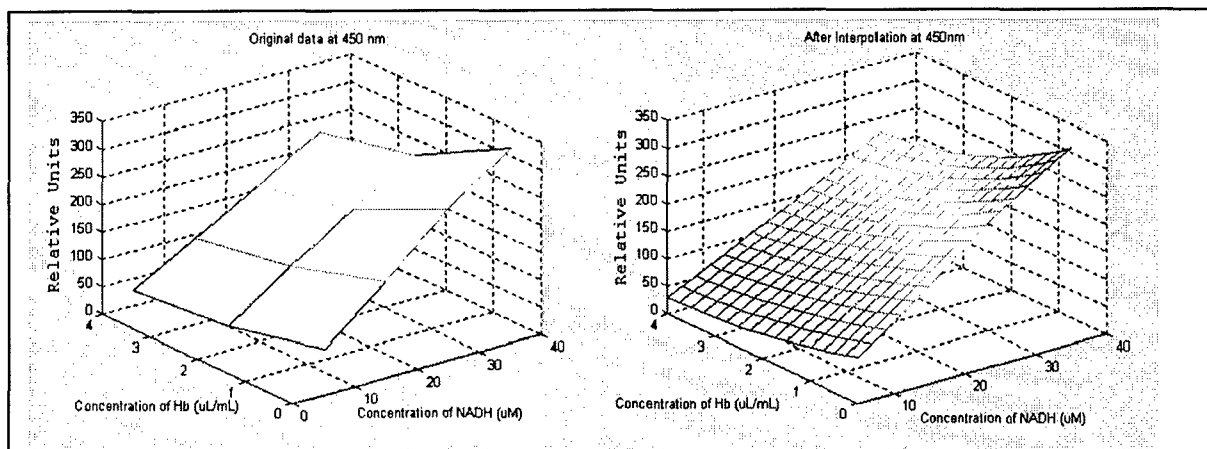
Changes in intramitochondrial NADH historically have been measured in cells and tissues by taking advantage of the fact that NADH is fluorescent when excited by near ultraviolet light. However, quantification of NADH concentrations in tissue has been complicated by the fact that any factors influencing fluorescence intensity, such as instrumentation variables and absorption of fluorescent light by compounds such as hemoglobin, will affect fluorescence measurements and consequently estimates of NADH concentration. We present a new method here that utilizes fiber-optic spectroscopy and a pre-calibration approach to estimate NADH concentration from small tissue volumes in the intact brain. We expect that this technique will allow our laboratory to extend our observations of the effects of mitochondrial toxins to studies in vivo.

The approach uses a 100  $\mu\text{m}$  bifurcated fused silica fiber-optic probe to deliver and collect NADH excitation and emission light respectively. Excitation light was provided by a pulsed nitrogen laser (337 nm) and emission spectra were acquired by a cooled photodiode array detector (325-625 nm). Examples of NADH emission spectra obtained from known concentrations of NADH are shown in figure 9. This figure shows the characteristic NADH emission spectrum with a maximum at 450nm and the linear relationship between NADH fluorescence and concentration.

However, the amplitude and shape of the NADH fluorescence emission spectra changes in the presence of hemoglobin. This effect is shown in Figure 9 for combinations of two NADH and hemoglobin concentrations. From a limited number of NADH /hemoglobin (Hb) concentration values, a three dimensional NADH/Hb X intensity surface was created at a single wavelength, and by interpolation a smoothed NADH/Hb know-value indexed amplitude surface was created. Examples of these surfaces are shown in Figure 10.



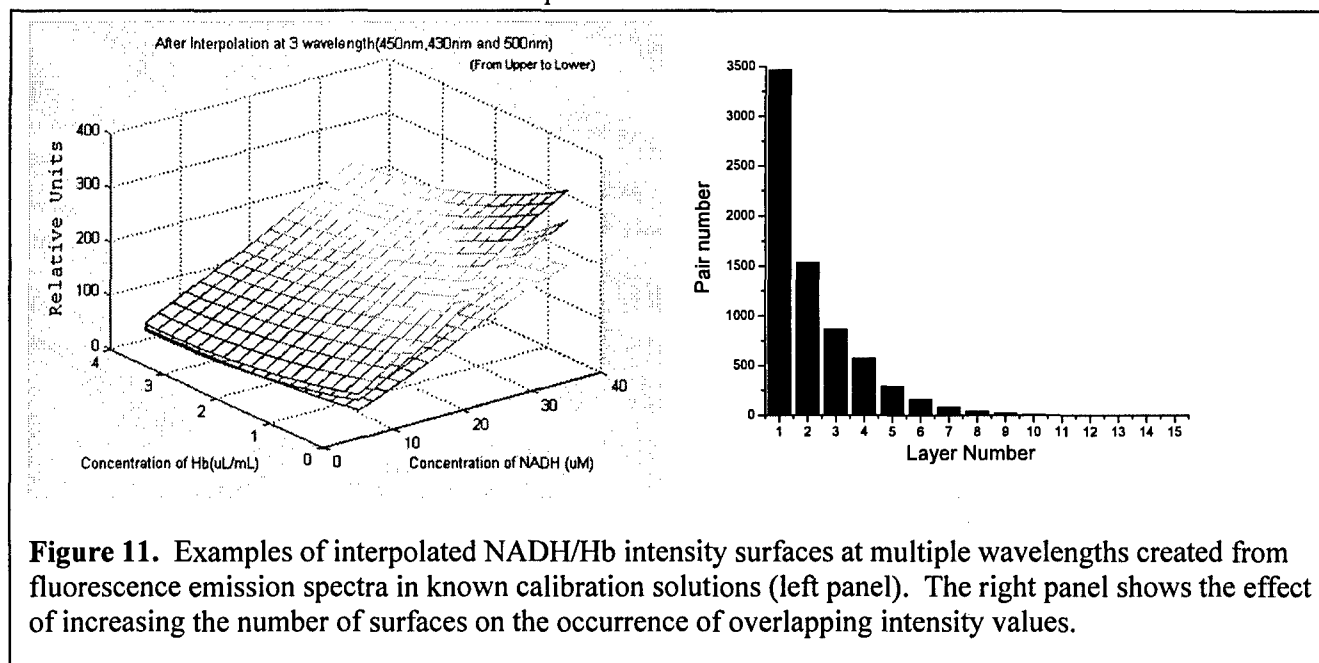
**Figure 9.** NADH emission spectra from solutions containing two different concentrations of NADH and hemoglobin. Note that the presence of hemoglobin reduced the fluorescence intensity in a wavelength dependent manner. The decrease in NADH fluorescence due to hemoglobin is consistent with the know absorption spectrum of this blood pigment.



**Figure 10.** Example of NADH/Hb intensity surface from known solutions of NADH and Hb (left) and an interpolated NADH/Hb known-value indexed intensity surface (right) for fluorescence intensity values obtained at 450 nm wavelength value.

From the fluorescence emission spectra, similar intensity surfaces were created for different emission wavelengths (Figure 11, left panel). Increasing the number of intensity surfaces by increasing the number of wavelengths sampled, had the effect of reducing the number of overlapping points among the intensity surfaces. This effect is shown in Figure 11 (right panel).

Using this approach we estimated the concentration of NADH in hippocampal slices obtained from rat brain both with (no cardiac perfusion) and without (cardiac perfusion with artificial cerebral spinal fluid) Hb. The probed indicated NADH concentration of  $9.3 \pm 2.3 \mu\text{M}$  ( $n=6$ ) in oxygenated brain slices devoid of Hb, and  $9.0 \pm 4.2 \mu\text{M}$  in Hb-containing brain slices. In hypoxic brain slices the NADH concentration increased to  $16.7 \pm 5.3 \mu\text{M}$  in Hb-free slices and  $16.3 \pm 5.5 \mu\text{M}$  in Hb-containing slices. In neither case was the difference between Hb-free and Hb-containing slices statistically significant. The observed values were similar to those reported by biochemical estimates from brain samples.



**Figure 11.** Examples of interpolated NADH/Hb intensity surfaces at multiple wavelengths created from fluorescence emission spectra in known calibration solutions (left panel). The right panel shows the effect of increasing the number of surfaces on the occurrence of overlapping intensity values.

## Key Research Accomplishments

- Continued experiments have not found evidence that mitochondrial depolarization, cytochrome c release or caspase activation contribute to neuronal death after toxin exposure
- New data suggests that Poly (ADP-ribose) polymerase (PARP) may contribute to mitochondrial loss of NADH after respiratory chain inhibition
- New data confirms the utility of using Enzyme-Dependent Fluorescence Recovery After Photobleaching (ED-FRAP) as measure of citric acid cycle dehydrogenase activity in functioning nervous tissue
- We report a method for quantifying mitochondrial NADH concentration in intact tissue using a fiber-optic pre-calibration approach

## Reportable Outcomes

S. Hagioka, M.D. Ginsberg, M.A. Perez-Pinzon, T.J. Sick. Poly(adp-ribose) polymerase inhibitors prevent loss of mitochondrial nadh and improve electrical recovery after anoxia in hippocampal SLICES Program No. 342.2. *2004 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2004.

Qiu, L., Zhao, W. and Sick, T.J. Quantitative analysis of brain NADH in the presence of hemoglobin using microfiber spectrofluorometry: a pre-calibration approach, *Computers in Biology and Medicine*, In Press.

## Conclusions

Mitochondrial toxins such as the Complex I inhibitor rotenone, cause neuronal cell death in the hippocampus in a dose-dependent manner. We have not been able to demonstrate involvement of mitochondrial permeability transition, release of cytochrome c, or caspase activation in this process. We have recently found, however, that Poly (ADP-ribose) polymerase (PARP) may contribute to mitochondrial dysfunction when the electron transport chain is inhibited. This data suggests that PARP may mediate neuronal cell death after toxin exposure either through cellular necrosis (ATP depletion) or an apoptosis pathway involving Apoptosis Inducing Factor (AIF) released from mitochondria. Future experiments will try to explore this possibility.

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